



Silver Nanoparticles Restore Root Growth of White Mustard Inhibited by Nitrate

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Abstract

Although the stimulatory effect of silver nanoparticles (Ag NPs) on root elongation has been studied, their effect in the presence of high nitrate levels remains unknown. In this study, we demonstrated that elongation of the primary root of white mustard (*Sinapis alba*) inhibited by nitrate was restored by the presence of Ag NPs. Ag NPs also increased root length under normal conditions in cultivation medium or water. RNA-seq of seedling roots revealed that the presence of Ag NPs affected the expression of glutamate receptors, genes involved in ethylene and auxin signaling, and in the ion homeostasis. Exposure to NO_3^- affected expression of nitrate transporters and genes involved in nitrate assimilation, borate transport, nicotianamine biosynthesis and cell wall processes. Despite significant differences in root growth in the presence of Ag NPs and NO_3^- , expression of genes involved in response to various stresses (e.g. pathogens, hypoxia, oxidative stress) and suberin and cutin biosynthesis increased under both treatments. Possible mechanisms responsible for the changes in root growth exposed to Ag NPs and NO_3^- were investigated in a series of the experiments. It revealed that the inhibitory effect of nitrate and the stimulatory effect of Ag NPs were related to the presence of ROS. The stimulatory effect was not specific to Ag NPs. Ionic Ag^+ had a similar effect.

Keywords RNA-seq · Nanomaterials · Seedling growth · Nitrate · *Sinapis alba*

Introduction

Silver (Ag) in the form of nanoparticles (NPs) or ions can positively affect plant growth. Increased germination rate and seedling growth (Mahakham et al. 2017), accelerated callus regeneration (Manickavasagam et al. 2019) or increased biomass growth and yield (El-Batal et al. 2016; Wang et al. 2013; Zou et al. 2025) are examples from the

literature. The beneficial effects of Ag NPs on plants were recently reviewed by Alfosea-Simón et al. (2025), Khina et al. (2024), and Khan et al. (2023). As Ag is not a biogenic compound, the question arises as to what mechanism is responsible for its stimulatory effects. According to Wang et al. (2020) a mild generation of reactive oxygen species (ROS) induced by Ag NPs was responsible for increased root elongation in *Arabidopsis*, while too high concentration of Ag NPs resulted in high levels of ROS that reduced root growth compared to control plants. Soaking of rice (*Oryza sativa*) seeds in Ag NPs suspension for 24 h increased germination rate, root and shoot growth and seedling weight. The authors revealed that seed exposure to Ag NPs increased seed water uptake and transcription of aquaporins *PIP1;1* and *PIP2;1*. Furthermore, soaking in Ag NPs suspension increased the α -amylase activity, soluble sugar content and the ROS levels in roots (Mahakham et al. 2017).

Silver ions are known to inhibit the action of ethylene (Beyer 1976). This gaseous phytohormone is involved in various physiological processes such as seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening, as well as in response to

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pathogens, wounding, hypoxia, ozone, chilling or freezing (Wang et al. 2002). In addition, ethylene promotes the biosynthesis and transport of auxin to the roots, resulting in the inhibition of cell elongation. Application of the ethylene precursor 1-aminocyclopropane-1- carboxylic acid (ACC) inhibits root growth, whereas Ag^+ ions interfered with the effect of ACC in *A. thaliana* plants (Růžicka et al. 2007). Therefore, another mechanism could be related to the inhibition of ethylene action.

Previously we observed that silver ions and NPs stimulate elongation of germinating white mustard (*Sinapis alba*, Brassicaceae) roots in culture medium (Landa et al. 2023). In this study, we observed that the medium containing a high concentration of nitrate inhibits root growth, whereas Ag NPs reversed this inhibition. Nitrate is one of the main sources of nitrogen for most plants and an important signaling molecule that influences plant architecture (Camut et al. 2021). To the best of our knowledge, the effect of Ag NPs and ionic Ag^+ on root growth in the presence of nitrate, and the mechanisms influencing growth inhibition and stimulation, have not yet been studied. We therefore decided to investigate the transcriptomic response of white mustard, a crop used as fodder, green manure and spice as well as a plant species commonly used in phytotoxicity assays (Mazur et al. 2016; Timmerer et al. 2020). We compared the gene expression profile of seedlings exposed to (a) water (control), (b) Ag NPs, (c) $\text{Ca}(\text{NO}_3)_2$ and (d) $\text{Ca}(\text{NO}_3)_2$ + Ag NPs with the aim to gain insight into the mechanisms responsible for these effects.

Materials and Methods

Seed Germination

Seeds of white mustard (*Sinapis alba*) var. Severka were obtained from Oseva Uni (Czech Republic) and germinated in Petri dishes filled with filter paper moistened with 5 mL of culture media. The pH of the culture media was set at 6.1–6.3. Seventeen seeds were placed into one dish. Root length was measured after 72 h of cultivation at room temperature in darkness. All experiments were conducted at least in triplicate. Results were expressed as a percentage of the control. Ag NPs (particle size < 100 nm, with PVP as a dispersant, purity 99.5% according to the manufacturer) were purchased from Sigma-Aldrich (MO, USA). Their characterization was previously described in Landa et al. (2023). All media containing Ag NPs were sonicated for 10 min to achieve suspension of nanoparticles. The salts used in the experiments were of analytical grade.

Experimental Design

Seedlings used for the RNA-seq were germinated under the following conditions: (1) water, (2) suspension of Ag NPs (1000 mg/L), (3) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (10 mM) dissolved in water, and (4) $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (10 mM) in suspension of Ag NPs (1000 mg/L). After three days of germination, the roots were separated from the seedlings, frozen in liquid nitrogen and stored at -80°C .

RNA-seq

Total RNA was extracted from the roots using RNeasy Plant Mini Kit (cat. no. 74904, Qiagen, Germany). Three samples per treatment (for one sample two to four roots were used) were used for the sequencing. Isolated RNA was treated with TURBO DNA-free™ Kit (cat. no. AM1907, Invitrogen, United States) to remove genomic DNA. Strand-specific cDNA libraries were constructed from polyA enriched RNA using KAPA mRNA HyperPrep kit (Roche) and sequenced on the Illumina NovaSeq X platform. The RNA-seq data used in this study are deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE274440). The sequencing resulted in at least 40 million 150 bps long read pairs. Rough reads were quality-filtered using Rcorrector and Trim Galore scripts (Song and Florea 2015) using default parameters. Levels of transcript expression (transcripts per million – TPM) were determined using Salmon (Patro et al. 2017) with parameters --posBias, --seqBias, --gcBias, --numBootstraps 30. Reference index was built from *S. alba* cds v 1.1 library obtained from Phytozome database. For more precise annotation, the closest *Arabidopsis* homolog for each *S. alba* transcript was determined by blastx. Visualization, quality control of data analysis and determination of differentially expressed genes were selected using sleuth (version 0.29.0) package in R (Pimentel et al. 2017). For estimation of statistical significance, genes that did not have at least 5 estimated counts in at least 25% of the samples were filtered out. Genes with $q\text{-value} \leq 0.05$ and $\log_2$ fold change ≥ 1 (upregulated) or ≤ -1 (downregulated) were considered to be significantly differentially expressed. GO analyses were performed using GO Term Enrichment tool accessible from (<https://pantherdb.org/webservices/go/overrep.jsp>; set up to test type: Fisher's Exact, correction: False Discovery Rate).

Quantitative Real-time PCR Analysis

The same RNA that was used for RNA-seq was reverse transcribed using M-MLV Reverse Transcriptase (RNase-H Minus, Point Mutant, Promega, United States), dT23dV primers and Protector RNase Inhibitor (Roche Applied Science, Germany). The resulting cDNA was 10-fold diluted and mixed with 5 μ L GoTaq qPCR Master Mix (Promega) and 0.5 mM specific primers to a final volume of 10 μ L. Sequences were amplified by qPCR: 45 cycles of 10 s at 95 °C for primer denaturation and 30 s at 60 °C for annealing and elongation using Light Cycler 480 (Roche Applied Science, Germany). Relative RNA concentration was calculated according to Livak and Schmittgen (2001) using two reference genes *EF1B* (*Sialb.0014s0086.1*) – f: CCACCTGCTAACTTTCCACAAC, r: AACCCCATTTCCTGATCGC; and *PP2A-2* (*Sialb.0088s0025.1*) – f: TGGAAACATGGCAGCGATTC, r: TCTGGTTCGACTTGTCTTGGAG. Primers used in this study: *ACO2* (*Sialb.0090s0048.1*) – f: TTCATCACTCGCCAGCAAAG, r: TGCTGTTGGGTTCAGTTCTG; *ERF72* (*Sialb.2278s0010.1*) – f: GCTTCAACCCACCAAACAAC, r: TTGAACGTACCAAGCCAGAC; *Sialb.3332s0003.1* – f: CCACAGATGAAGGAGCCTAATG, r: TGAACAGCACGGCATCTTTG; *Sialb.0039s0124.1* – f: ATGGAGATGGCGGATACTGTTC, r: TGCCACGCAATCAACACTTC.

Statistical Evaluation

Length of the roots is presented as the mean of at least three independent biological replicates $\pm$ standard deviation per treatment. The error bars in the figures indicate the standard deviation of the measured root lengths. One-way ANOVA analysis of variance with the Tukey HSD multiple comparison test was used to evaluate the null hypothesis of mean compression between multiple sets. Statistically different values at the probability level $p < 0.05$ are indicated by the different letter in figures.

Results and Discussion

Root Elongation Under Ag NPs and NO_3^- Exposure

Root elongation of white mustard was inhibited in the presence of nitrates. KNO_3 , NH_4NO_3 (both at 3 mM concentration) and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (2 mM concentration) reduced root length by about 50% compared to control plants germinating in the water (Fig. 1). Conversely, K_2HPO_4 , $\text{NH}_4\text{H}_2\text{PO}_4$ and $\text{Ca}_3(\text{PO}_4)_2$ did not inhibit root growth indicating that the presence of K^+ , NH_4^+ and Ca^{2+} ions at the

same concentrations as in the respective nitrates was not responsible for the inhibition of root growth (Fig. 1). Negative effect of high nitrate concentration on primary root length was observed previously in *Arabidopsis* (Linkohr et al. 2002; Vidal et al. 2013). For further experiments, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ was used as nitrate source. Based on the screening results, Ag NPs were used at a concentration of 1000 mg/L (Fig. 2a). Figure 2b shows the effect of Ag NPs on the root elongation of seeds germinating in the presence of 2 and 10 mM nitrate. The seedlings germinated in the water containing Ag NPs also increased root length. Finally, although that no statistical difference was found for nitrate concentrations tested, the 10 mM concentration showed a slightly higher potential to inhibit root growth and was used in further experiments. The phenotype of mustard seedlings exposed to Ag NPs and nitrate is shown in Fig. 3. Stimulatory effect was not specific to Ag NPs. Ionic Ag^+ (applied as AgNO_3) also stimulated root elongation at the concentration of 1 and 10 mg/L while 100 mg/L had a negative effect (Supplementary Fig. S1).

Impact of Ag NPs and NO_3^- on Gene Expression

Exposure to $\text{Ca}(\text{NO}_3)_2$ changed the expression of the largest number of genes (574 up- and 638 down-regulated), followed by the combination of $\text{Ca}(\text{NO}_3)_2$ + Ag NPs (616 up- and 444 down-regulated) and Ag NPs (401 up- and 275 down-regulated). Interestingly, the overlap between plants exposed to Ag NPs and $\text{Ca}(\text{NO}_3)_2$ was much higher for up-regulated (red dots) than for down-regulated genes (blue dots) (Fig. 4). Surprising is also only small number of genes that were regulated in opposite direction in both treatments (yellow dots). The full list of differentially expressed genes under each treatment is shown in Supplementary Table S1.

Ethylene Signaling and Biosynthesis

Exposure to Ag NPs alone as well as in combination with NO_3^- down-regulated the number of genes involved in ethylene biosynthesis and signaling (Supplementary Table S2). Ethylene inhibits primary root growth in interaction with other phytohormones and promotes the biosynthesis and transport of auxin to the roots, resulting in inhibition of cell elongation (Qin et al. 2019). Exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) inhibited root growth, while Ag^+ ions (inhibitor of ethylene perception) interfered with the effect of ACC in *A. thaliana* plants (Růžicka et al. 2007). In our study, the presence of Ag NPs decreased the expression of ACC synthase *ACSII* and the expression of ACC oxidases *ACO1* and

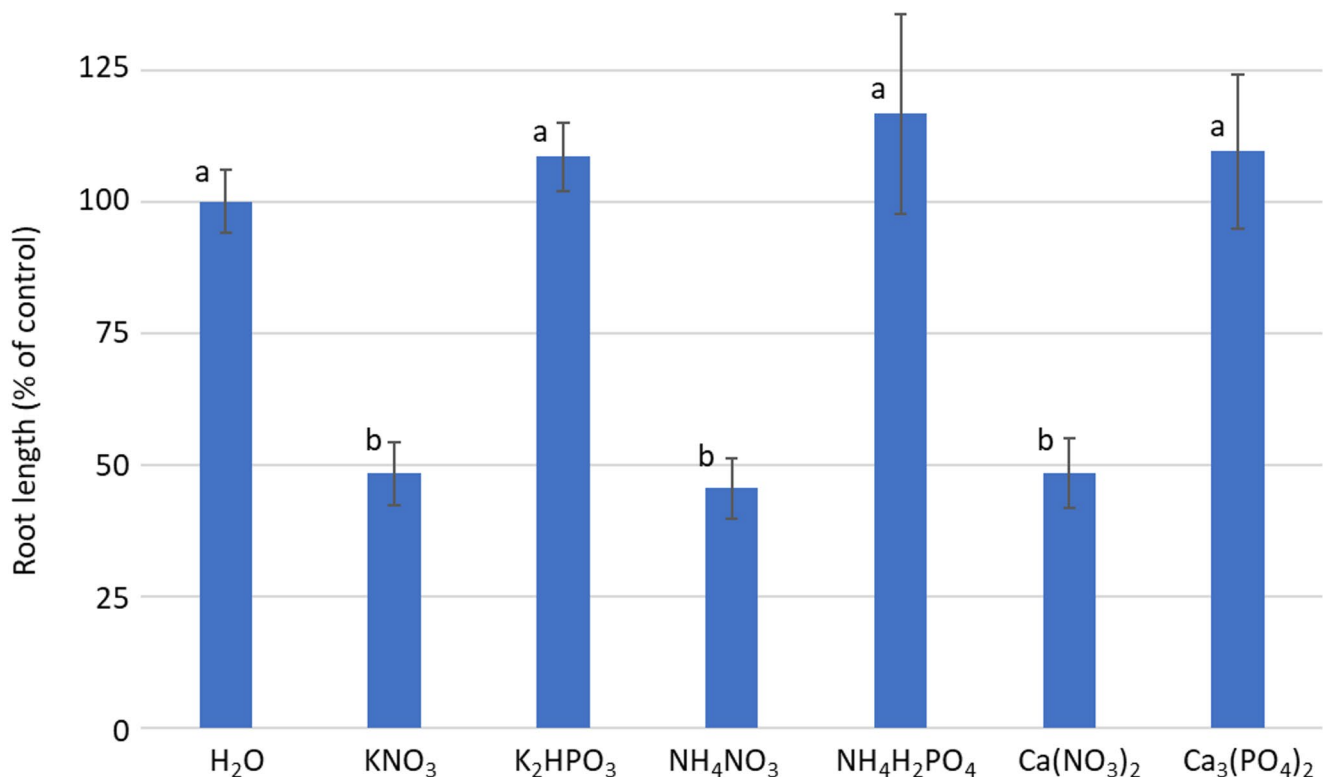


Fig. 1 Effect of various nitrate and phosphate salts on the root length of white mustard seedlings after three-day cultivation. KNO₃, NH₄NO₃ in 3 mM concentration and Ca(NO₃)₂·4H₂O in 2mM concentration were used. K₂HPO₄, NH₄H₂PO₄, and Ca₃(PO₄)₂ in concentration cor-

responding concentrations of K⁺, NH₄⁺, and Ca²⁺ ions as in case of nitrate salts. Length of the roots is expressed as % of the control (H₂O as culture medium). Different letters indicate statistically significant difference (one-way ANOVA, $p < 0.05$) between treatments ($n = 3$)

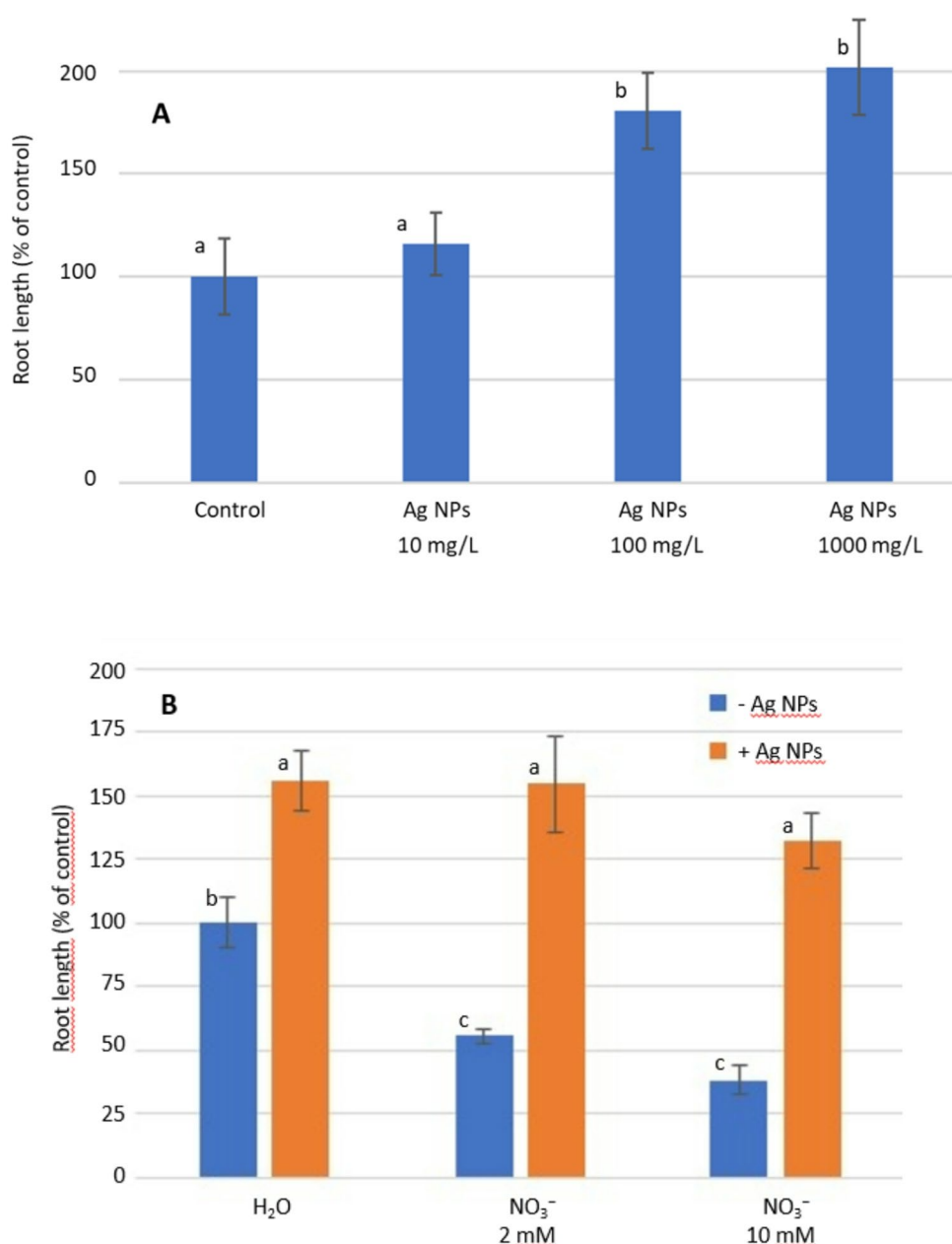
ACO2 (Table 1). ACC synthases catalyze the synthesis of ACC and ACC oxidases convert ACC to ethylene (Pattyn et al. 2021). Therefore, the down-regulation of genes involved in ethylene biosynthesis and several other genes involved in ethylene signaling pathway indicates that positive effect of Ag NPs on the root growth could be attributed to the negative effect of silver on ethylene signaling cascade. One would then expect that the inhibition of root growth in presence of NO₃[−] would be associated with increased expression of these genes. However, exposure to NO₃[−] only increased the transcription of *ACS7* (and decreased *ACS5*), and two ethylene-responsive element binding proteins *RAP2.3* and *RAP2.6 L*. Application of 1 μM CoCl₂ (Co²⁺ ions are the inhibitor of ethylene biosynthesis; Lau and Yang 1976) slightly increased root length compared to the untreated control but had no positive effect in the presence of NO₃[−] in our study (Supplementary Fig. S2). Together, the results indicate that reduced ethylene synthesis/signaling in the presence of Ag NPs may play a role in the root growth stimulation but it seems does not appear to explain the effect of Ag NPs in presence of high nitrate. *RAP2.6 L* (also known as *ERF113*) is close homolog of *ERF115*. Lakehal et al. (2020) showed that *ERF115* functions as a repressor of adventitious root initiation via cytokinin signaling and that

it is transcriptionally activated by jasmonate. As mentioned above, *RAP2.6 L* and *RAP2.3* (known also as *ERF72*) were up-regulated in presence of NO₃[−] (while down-regulated by Ag NPs). It indicates that these genes could play a role in root growth under NO₃[−] and Ag NPs but question remains whether they could affect primary root elongation in *S. alba*.

Auxin Signaling and Biosynthesis

Inhibition of root elongation is synergistically influenced by ethylene and auxin. Ethylene increases auxin response in the root elongation zone (Muday et al. 2012). In our study, the presence of Ag NPs decreased the expression of the ethylene-responsive *YUC9*, which is associated with auxin biosynthesis as well as expression of *ARGOS* (auxin-regulated gene involved in organ size) and *SAUR52*, *SAUR69*, and *SAUR76* genes from SAUR-like auxin-responsive protein family. On the other hand, exposure to NO₃[−] increased expression of some SAURs and decreased expression of auxin efflux carrier *PIN2* and intracellular auxin carriers *PILS3* and *PILS5* (Table 2). Strader et al. (2009) suggested that Ag⁺ ions can promote auxin efflux impudently on the ethylene action. In their study, the reduction of *Arabidopsis* root growth by exogenous application of IAA was partially

Fig. 2 Root length of white mustard seedlings after three-day cultivation (A) in the presence of Ag NPs (control=50% Hoagland's solution) (B) in the presence of various nitrate concentrations and presence/absence of Ag NPs (1000 mg/L). Different letters indicate statistically significant difference (one-way and two-way ANOVA, $p < 0.05$) between treatments ($n = 3$)



restored by AgNO₃ but not by another inhibitor of ethylene signaling AVG (2-aminoethoxyvinyl glycine). Therefore, it seems possible, that Ag NPs and NO₃⁻ affected root growth via auxin signaling. However, Ag NPs did not reverse root inhibition after exogenous application of 1 μM IAA in our study (Supplementary Fig. S3).

Glutamate Signaling

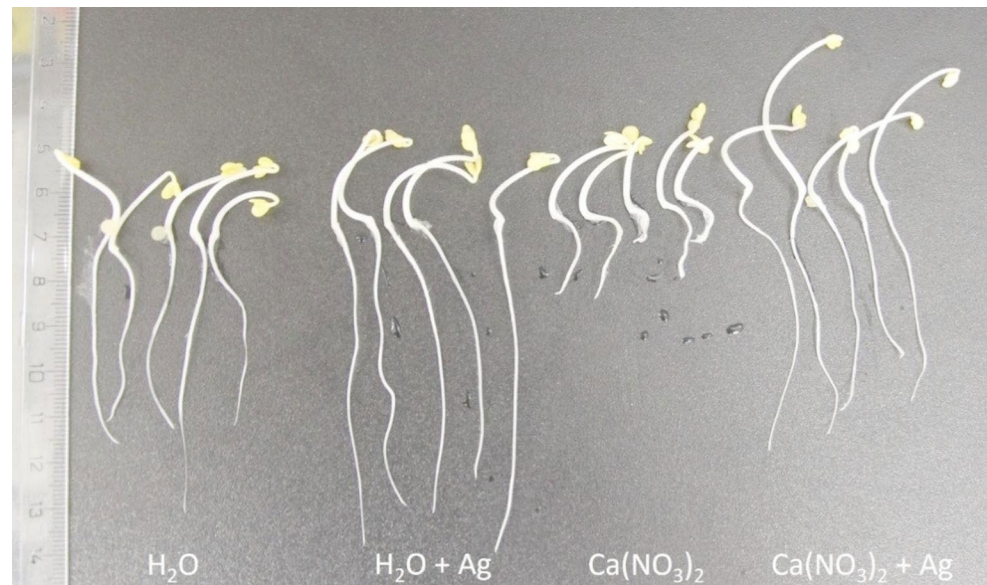
Presence of Ag NPs increased the expression of glutamate receptors *GLR1.1* and *GLR1.3* while under high NO₃⁻ glutamate receptor *GLR2.4* was down-regulated. Since 100 μM L-glutamate inhibited *Arabidopsis* root length to 20%

of control (Walch-Liu et al. 2006), we hypothesized that the effect of Ag NPs might be related to glutamate signaling. However, L-glutamate at 0.1 and 1 mM concentration had only weak negative effect on root growth (Supplementary Fig. S4). Therefore, we ruled out possible interaction of Ag NPs and glutamate signaling.

Nitrate Transport and Assimilation

Exposure to high nitrate unsurprisingly affected the expression of genes associated with nitrate transport and assimilation (Supplementary Table S3). Expression of these genes was mostly changed similarly under NO₃⁻ exposure

Fig. 3 Effect of Ag NPs (1000 mg/L) and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ in 10 mM concentration on root length of white mustard seedlings after three-day cultivation



and co-exposure to NO_3^- and Ag NPs. It indicates that Ag NPs did not affect nitrate assimilation. Interesting seems up-regulation of nodulin/glutamine synthase-like protein (*NODGS*), which occurred in the case of Ag NPs exposure and Ag NPs and NO_3^- co-exposure but not in the presence of NO_3^- . Down-regulation of this gene by iRNA in *Arabidopsis* resulted plants with short main root, reduced meristematic activity and disrupted root cap development in *Arabidopsis* (Doskočilová et al. 2011).

Ion Homeostasis

Exposure to Ag NPs and to NO_3^- affected the expression of several cation exchangers and transporters (Supplementary Table S4). Interestingly, the presence of NO_3^- led to a strong upregulation of the high-affinity K^+ transporter *HAK5*, while this gene remained unaffected when the seedlings were exposed to NO_3^- together with Ag NPs (slightly down-regulated). Furthermore, the presence of NO_3^- increased expression of *Sialb.0150s0011.1* and *Sialb.0150s0012.1* similar to *Arabidopsis* high-affinity K^+ transporter *HKT1* while co-exposure to NO_3^- and Ag NPs increased their expression only insignificantly. Nitrate is able to alter electrical potential and pH gradient in plasma and intracellular membranes (Sze and Chanroj 2018). Moreover, hydroxide ions are produced during nitrate assimilation. To maintain cytosolic pH homeostasis, OH^- ions are neutralized with organic acids or sequestered to the rhizosphere (Raven and Smith 1976). Marschner and Römheld found increased pH values in the rhizosphere of maize when nitrogen source was nitrate (Marschner and Römheld 1983). In our experiment, the highest pH (7.17) of culture medium was observed in presence of NO_3^- after three days of cultivation. Interestingly, when plants germinated in the presence of NO_3^- together

with Ag NPs, final pH was 6.60. Also germination in water without Ag NPs increased pH more (6.83) than germination in presence of Ag NPs (pH=6.49). It shows that the presence of Ag NPs slowed down the increase in medium pH. For the cell growth, activation of cell wall loosening enzymes is required for cell wall remodeling. Acidification of the apoplast is considered as a necessary step for the action of these enzymes. Acidification is achieved via activation of proton pumps through auxin activity (Barbez et al. 2017). Overexpression of SAUR 19 resulted in proton pump activation and apoplast acidification in *Arabidopsis* (Spartz et al. 2014). As mentioned above, the presence of Ag NPs and NO_3^- affected expression of several SAUR genes (Table 2). Further, Staal et al. demonstrated that application of ACC alkalinized the apoplast of *Arabidopsis* roots and reduced their growth. Cell elongation was partially restored by fusaric acid, an activator of the plasma membrane H^+ -ATPase (Staal et al. 2011). Altogether, we assumed that inhibition of root growth by high concentration of NO_3^- and its restoration by Ag NPs was related to ion homeostasis and pH changes. Since, Cl^- ions are (similarly to NO_3^-) able to change the membrane electric potential and pH gradient (Sze and Chanroj 2018), we exposed plants to 100 mM KCl. However, Ag NPs were unable to recover root growth (Supplementary Fig. S5). Similarly, adjusting the cultivation media to pH to 4.5 and 9 did not affected root growth in presence of NO_3^- (Supplementary Fig. S6). Exposure to NO_3^- down-regulated vacuolar iron transporters (Supplementary Table S4). The presence of NO_3^- also increased the expression of genes involved in nicotianamine biosynthesis. Nicotianamine is a strong metal chelator that is important for internal transport of metal ions (Carrillo and Borthakur 2021). It indicates that the presence of NO_3^- increased demand for Fe, which is consistent with previous findings

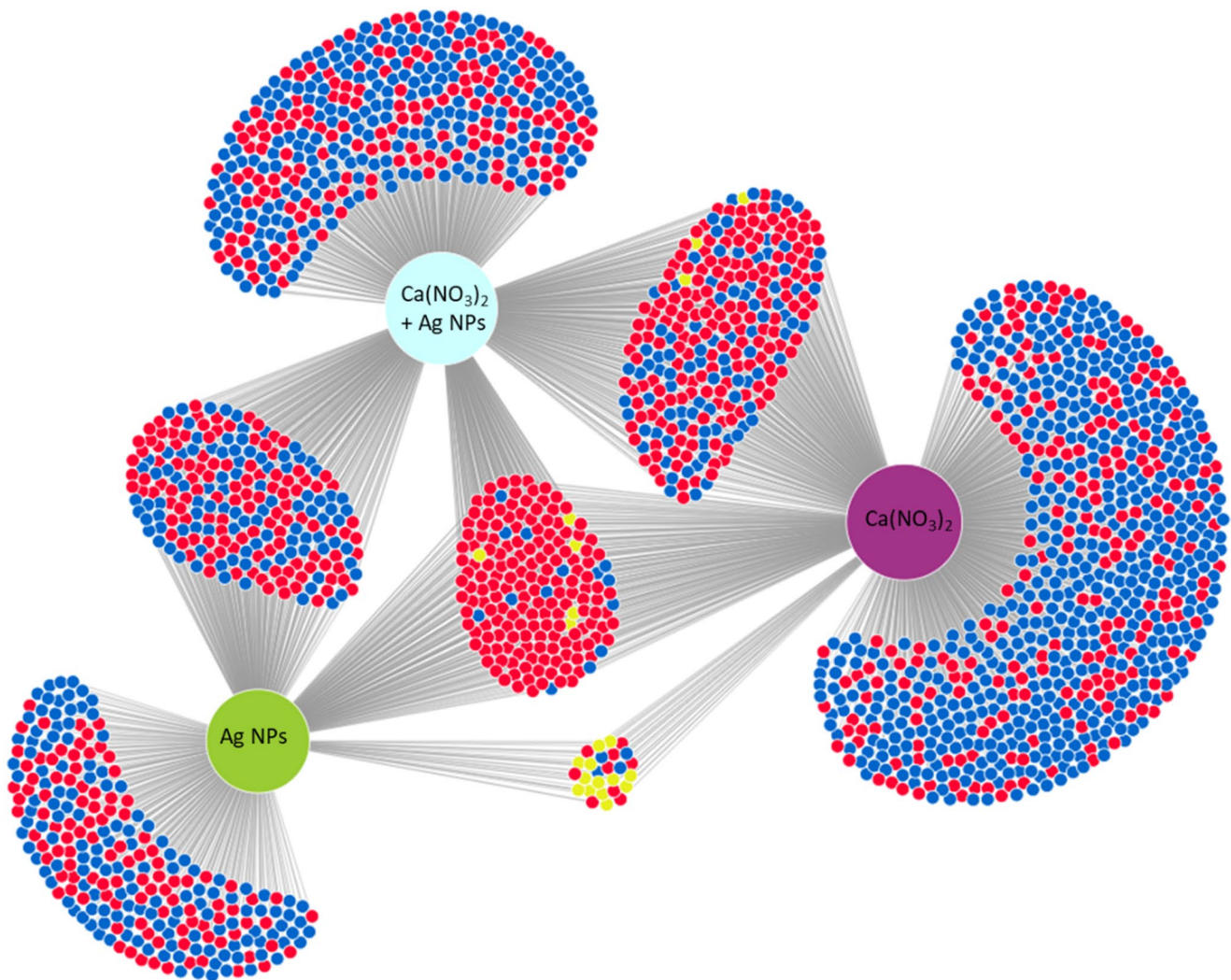


Fig. 4 Changes in gene expression after three-day exposure to Ag NPs, high nitrate and combination of both treatments in comparison with untreated control. Up-regulated genes are represented by red dots, down-regulated genes by blue dots and yellow dots represents

genes up-regulated under one treatment and down-regulated in other treatment. The lines represent the connection between genes and the respective treatment

that nitrate increases Fe-deficiency phenotype (Sun et al. 2021). On the other hand, presence of Ag NPs resulted in down-regulation of iron-regulated transporters *IRT1* and *IRT2* (Supplementary Table S4), which are up-regulated in response to iron-limited conditions (Vert et al. 2001).

Boron

Presence of NO_3^- up-regulated two boron transporters *BOR1* and *BOR4*. Interestingly, the expression of these transporters was not up-regulated when seedlings were co-exposed to NO_3^- and Ag NPs. *BOR1* is key transporter induced under boron deficiency. Previous studies demonstrated that *bor1* mutants are sensitive to increased nitrate concentration and growth of their root is reduced under high NO_3^- (Wang et al. 2021). Moreover, the inhibition of cell

elongation in *Arabidopsis* roots caused by boron deprivation was mitigated by inhibitors of ethylene action (aminoethoxyvinylglycine and silver ions) as well as by antagonist (α -(phenylethyl-2-oxo)-indoleacetic acid) of auxin receptor TIR1 (Camacho-Cristóbal et al. 2015). Therefore, we hypothesized that boron application could mitigate the effect of nitrate. However, boron at 10 and 100 μM concentrations, applied in the form of H_3BO_3 , had no effect on the root growth in the presence of NO_3^- (Supplementary Fig. S7).

Stress

Although Ag NPs stimulated root growth while NO_3^- repressed it, presence of both substances increased the expression of a high number of genes involved in response

Table 1 Selected genes involved in ethylene biosynthesis and signaling differentially expressed under Ag NPs and NO₃[−] treatment (complete list is in supplementary table S2). Genes significantly up-regulated (qval < 0.05; fold-change (log2) > 1) are highlighted by red and genes significantly down-regulated by blue color

<i>A. thaliana</i> gene ID	<i>A. thaliana</i> symbol	<i>A. thaliana</i> description	<i>S. alba</i> gene ID	Fold change (log2) Ag NPs	Fold change (log2) Ca(NO ₃) ₂	Fold change (log2) Ag NPs + Ca(NO ₃) ₂
AT3G16770.1	RAP2.3, ATEBP, ERF72, EBP	ethylene-responsive element binding protein	Sialb.0368s0018.1	-2.91	0.12	-1.90
			Sialb.2278s0010.1	-2.67	2.18	-1.21
			Sialb.2833s0004.1	-2.18	0.32	-1.29
AT5G13330.1	Rap2.6L	related to AP2 6L	Sialb.0006s0130.1	-2.31	0.83	-1.05
			Sialb.0096s0020.1	-0.35	2.57	0.76
			Sialb.1207s0012.1	-0.81	2.16	1.34
AT5G21120.1	EIL2	ETHYLENE-INSENSITIVE3-like 2	Sialb.0050s0033.1	-2.78	0.68	-1.73
			Sialb.0564s0021.1	-6.05	1.85	-1.50
AT2G19590.1	ACO1, ATACO1	ACC oxidase 1	Sialb.0005s0053.1	-1.52	0.26	-3.65
AT1G62380.1	ACO2, ATACO2	ACC oxidase 2	Sialb.0090s0048.1	-1.82	0.64	-2.16
AT5G65800.1	ACS5, CIN5, ETO2, ATACS5	ACC synthase 5	Sialb.0205s0091.1	0.70	-2.01	-0.91
			Sialb.0499s0013.1	0.47	-1.71	-0.52
			Sialb.1229s0010.1	0.46	-1.83	-0.64
AT4G26200.1	ACS7, ATACS7	ACC synthase 7	Sialb.0043s0057.1	0.41	1.86	0.81
AT4G08040.1	ACS11	ACC synthase 11	Sialb.0240s0026.1	-2.07	-0.86	-1.65
AT1G04310.1	ERS2	ethylene response sensor 2	Sialb.0073s0047.1	-2.41	1.17	-1.57
			Sialb.0615s0059.1	-3.67	1.08	-1.44
AT3G23150.1	ETR2	Signal transduction histidine kinase, hybrid-type, ethylene sensor	Sialb.2064s0002.1	-1.72	0.69	-1.46

to various stress conditions such as oxidative stress, hypoxia, fungi and bacteria (results of GO enrichment analysis for each treatment are given in Supplementary Table S5). Further, genes involved in suberin and cutin (substances that act as a barrier in the exchange of water, solutes and gases and as a protection against abiotic and biotic stresses (Pollard et al. 2008)) were up-regulated under both treatments. Exposure to Ag NPs was associated with oxidative stress and ROS production in plants. This resulted in negative effects such as ion leakage and cell death (Homaei and Ehsanpour 2016), though mild ROS production was associated with stimulating the growth of *Arabidopsis* roots (Wang et al. 2020).

Reactive Oxygen Species

Consistent with our results, nitrate at 5 mM concentration inhibited the growth of *Medicago truncatula* primary root. The authors of the study demonstrated that the inhibition was concerned with reduced accumulation of ROS in the root tips and was recovered with external application of H₂O₂ (Zang et al. 2020). In our conditions, H₂O₂ at 10 mM increased root length by 40% compared to the control (Fig. 5a). Further, H₂O₂ at 10 mM concentration partially restored root elongation in presence of nitrate (Fig. 5b). Moreover, potassium iodide (KI), a H₂O₂ scavenger (Junglee et al. 2014), decreased root growth of white mustard at 10 mM (Fig. 5c), while Ag NPs partially recovered its growth (Fig. 5d). It indicates that effect of NO₃[−] and Ag NPs relates

with ROS activity. ROS play essential role of in root growth and their importance is comparable to plant hormones. They modify structure of cell walls and size of the meristematic zone by regulating the cell cycle (Tsukagoshi 2016). Zang et al. showed that NO₃[−] altered activity of respiratory burst oxidase homologues (RBOHs), class III peroxidases and superoxide dismutases in *M. truncatula* root (Zang et al. 2020). In our experiment, both Ag NPs and NO₃[−] affected expression of many peroxidases (Supplementary Table S6). In some cases (e.g. *PRX34*, *AT2G35380*, *AT1G68850* and *AT5G14130*), there was similar effect despite different treatment. However, some transcripts were up- (*PRX47* and *PRX71*) or down-regulated (*GPX2*, *AT3G01190*, *PRX52*, *PRX56*, and *PRX64*) only in presence of NO₃[−] and some down-regulated (*PRX44* and *AT5G19890*) in presence of Ag NPs with and without NO₃[−]. On the other hand, the transcription of superoxide dismutases or RBOHs was not affected in our conditions.

Quantitative Real-time PCR Analysis

Although RNA-seq is considered a method that is not affected by reproduction variability and bias as some microarrays (Coenye 2021), we selected four genes representing highly and slightly up- and down-regulated transcripts under all three treatments to validate the RNA-seq data by quantitative real-time PCR analysis. The expression of the genes measured by both methods showed good agreement (Table 3). It indicates that data from RNA-seq is reliable.

Table 2 Genes involved in auxin biosynthesis and signaling differentially expressed under Ag NPs and NO_3^- treatment. Genes significantly up-regulated ($q\text{val} < 0.05$; fold-change ($\log_2$) > 1) are highlighted by red and genes significantly down-regulated by blue color

<i>A. thaliana</i> gene ID	<i>A. thaliana</i> symbol	<i>A. thaliana</i> description	<i>S. alba</i> gene ID	Fold change ($\log_2$) Ag NPs	Fold change ($\log_2$) $\text{Ca}(\text{NO}_3)_2$	Fold change ($\log_2$) Ag NPs + $\text{Ca}(\text{NO}_3)_2$
AT5G20820.1	SAUR76	SAUR-like auxin-responsive protein family	Sialb.0050s0055.1	-2.44	3.45	-0.06
			Sialb.1230s0018.1	-3.93	1.64	-2.57
AT3G12830.1	SAUR72	SAUR-like auxin-responsive protein family	Sialb.0095s0021.1	-0.09	1.75	2.31
			Sialb.2598s0001.1	0.69	1.33	1.58
AT2G36210.1	SAUR45	SAUR-like auxin-responsive protein family	Sialb.0203s0007.1	0.00	1.88	0.74
AT1G16510.1	SAUR41	SAUR-like auxin-responsive protein family	Sialb.0844s0017.1	0.86	3.07	2.93
AT5G10990.1	SAUR69	SAUR-like auxin-responsive protein family	Sialb.0025s0285.1	-2.12	1.26	-0.58
AT1G75590.1	SAUR52	SAUR-like auxin-responsive protein family	Sialb.0102s0043.1	-4.69	2.00	-1.11
AT5G53590.1	SAUR30	SAUR-like auxin-responsive protein family	Sialb.1188s0007.1	0.66	0.63	1.35
AT3G60690.1	SAUR59	SAUR-like auxin-responsive protein family	Sialb.1417s0003.1	0.76	1.23	1.77
AT4G37390.1	YDK1, GH3.2, BRU6, GH3-2, AUR3	Auxin-responsive GH3 family protein	Sialb.5505s0001.1	-1.02	2.31	0.62
AT4G23980.1	ARF9	auxin response factor 9	Sialb.1449s0013.1	0.25	-1.85	-1.19
AT3G59900.1	ARGOS	auxin-regulated gene involved in organ size	Sialb.0079s0079.1	-1.98	1.45	-2.15
			Sialb.1735s0011.1	-2.98	0.31	-1.66
AT5G57090.1	EIR1, WAV6, ATPIN2, PIN2, AGR, AGR1	Auxin efflux carrier family protein	Sialb.0198s0318.1	0.11	-1.90	-0.19
AT2G17500.4	PILS5	Auxin efflux carrier family protein	Sialb.0225s0006.1	-0.14	-2.78	-1.09
			Sialb.0383s0083.1	-0.18	-2.92	-2.36
AT1G76520.2	PILS3	Auxin efflux carrier family protein	Sialb.0688s0019.1	0.90	-2.89	-0.96
AT1G04180.1	YUC9	YUCCA 9	Sialb.0073s0057.1	-1.48	-0.26	-1.59
			Sialb.0074s0124.1	-2.14	0.46	-1.52
AT3G23050.1	IAA7, AXR2	indole-3-acetic acid 7	Sialb.0122s0004.1	-1.13	0.10	-1.89
			Sialb.0619s0014.1	-1.22	0.17	-1.69
AT4G32280.1	IAA29	indole-3-acetic acid inducible 29	Sialb.0976s0023.1	-1.95	0.31	-2.23
AT1G44350.1	ILL6	IAA-leucine resistant (ILR)-like gene 6	Sialb.0195s0010.1	-0.03	0.45	1.53
AT1G51760.1	IAR3, JR3	peptidase M20/M25/M40 family protein	Sialb.0517s0023.1	-0.52	-1.52	-0.51
AT1G05700.1		Leucine-rich repeat transmembrane protein kinase protein	Sialb.0629s0023.1	0.58	-1.95	-1.60
AT1G24180.1	IAR4	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein	Sialb.0199s0031.1	0.40	2.12	1.97

Conclusions

Silver in the form of nanoparticles and ions was able to reverse the negative effect of high nitrate on root growth of white mustard seedlings. Based on transcriptional profiling, we proposed several mechanisms responsible for this effect. One possible target of Ag NPs (Ag^+ ions released from NPs) is ethylene signaling and biosynthesis, as Ag NPs negatively affected genes involved in this pathway. This hypothesis is supported by the fact that silver is a known inhibitor of ethylene perception. On the other hand, presence of NO_3^- up-regulated only a few genes involved in ethylene signaling and the application of Co^{2+} (another ethylene antagonist) did not reverse the inhibition of root growth in presence of NO_3^- . Another potential target appears to be auxin signaling, although that Ag NPs did not alleviate root growth inhibition in presence of exogenous IAA. Further we suggested, that the presence of Ag NPs stabilized the ionic homeostasis disturbed by NO_3^- . However, additional experiments with KCl, acidic and basic pH and boron did not indicate

that ionic imbalance was the original cause affecting root growth in the presence of Ag NPs and nitrate. Finally, as Zang et al. (2020) linked the effect of root growth inhibition by nitrate with changes in ROS production/decomposition, we also tested this possibility. H_2O_2 partially alleviated the inhibition caused by NO_3^- and Ag NPs partially restored the growth of roots inhibited by KI. It demonstrates that both NO_3^- and Ag NPs influence primary root growth of white mustard via ROS. Peroxidases could be the players in this process as the expression of many of them was influenced by Ag NPs and nitrate. Since the stimulatory effect was not specific to Ag NPs, but was also observed in the presence of ionic Ag^+ , we assume that ions released from NPs act on the plants.

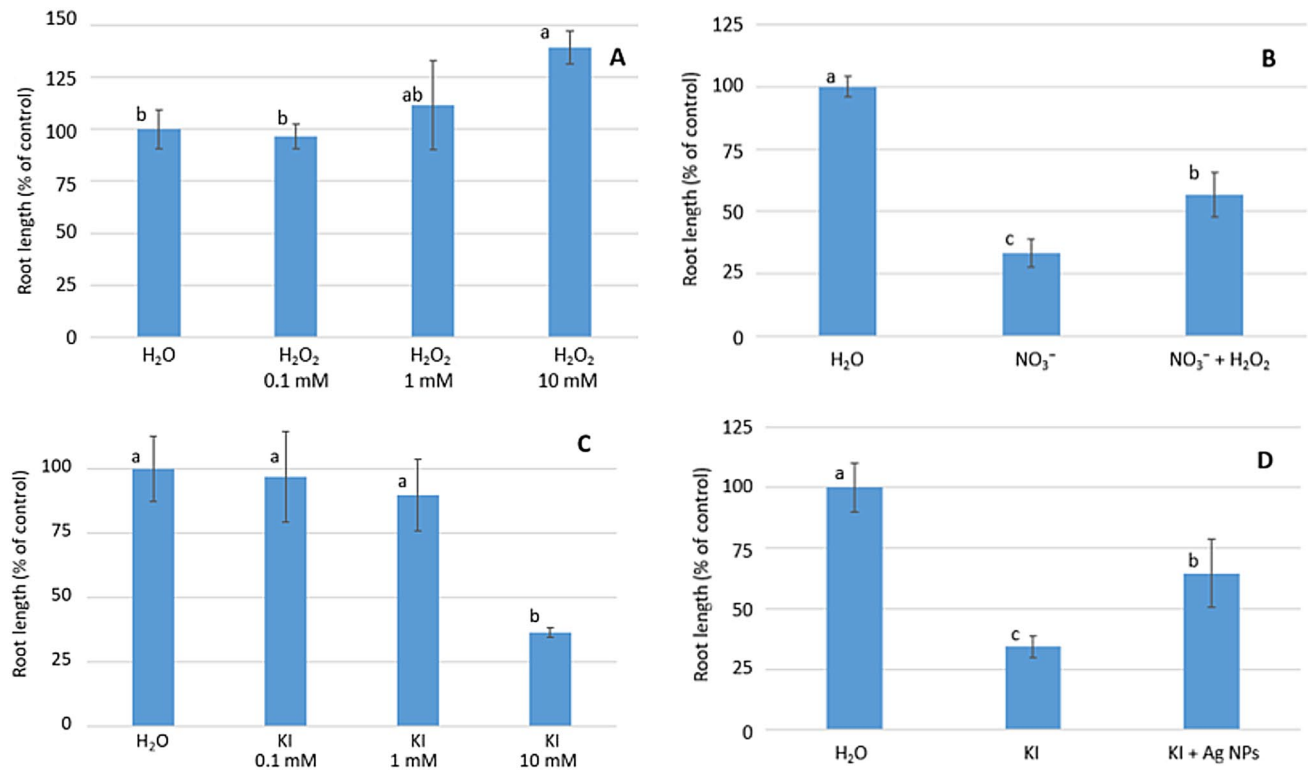


Fig. 5 Root length of white mustard seedlings after three-day cultivation (A) effect of different H₂O₂ concentrations (B) effect of 10 mM NO₃⁻ and 10 mM H₂O₂ (C) effect of different KI concentrations (D)

effect of 10 mM KI and Ag NPs (1000 mg/L). Different letters indicate statistically significant difference (one-way ANOVA, $p < 0.05$) between treatments ($n = 3-5$)

Table 3 Validation of transcription profiles by RT-qPCR

S. alba gene ID	Fold change (log2)							
	Sialb.0090s0048.1		Sialb.2278s0010.1		Sialb.3332s0003.1		Sialb.0039s0124.1	
Gene name	ACO2		ERF72		NODGS		WALK11	
	RNA-seq	qPCR	RNA-seq	qPCR	RNA-seq	qPCR	RNA-seq	qPCR
Ag NPs	-1.82	-2.21	-2.67	-3.36	2.03	1.37	4.44	3.13
Ca(NO ₃) ₂	0.64	0.31	2.18	1.83	1.52	0.89	1.74	1.07
Ca(NO ₃) ₂ + Ag NPs	-2.16	-2.28	-1.21	-1.39	2.35	2.54	5.04	4.10

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Author Contributions Přemysl Landa: Experimental design, manuscript writing, data analysis, RNA extraction. Karel Müller: Processing of RNA-seq data, statistical analysis. Sylva Přerostová: Quantitative real-time PCR. Kateřina Mořková: phytotoxicity assays. Petr Soudek: funding acquisition.

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Data Availability Data from RNA-seq are available at Gene Expression Omnibus accession no. GSE274440.

Declarations

Conflict of interest Authors declare no competing interests.

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